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13. ABSTRACT (Maximum 200 Words)

Carcinoembryonic antigen (CEA) is a highly glycosylated protein that is overexpressed in many human cancers including, breast cancer. Recent studies suggest that overexpression of CEA promotes tumorogenesis by inhibiting cell differentiation, and by preventing anoikis, a type of apoptotic program which destroys cells that lose contact with the extracellular matrix. If and how CEA promotes breast cancer remains to be determined. Our hypothesis is that if overexpression of CEA is linked to breast cancer progression, downregulation of CEA may promote apoptosis or alter the expression of genes that are involved in apoptosis and cell cycle/proliferation. In order to test this hypothesis, we employed RNA interference (RNAi) to downregulate CEA mRNA. RNAi is a recently discovered phenomenon, which induces sequence-specific gene silencing in response to exogenous double-stranded RNA (dsRNA). Our data indicate that among a variety of siRNAs tested, a chemically synthesized siRNA homologous to CEA (nucleotide 402-422) has been most effective in suppressing the expression of CEA. We have also developed transcription condition that permits T7 RNA polymerase catalyzed incorporation of 8-N_3ATP into RNA. 8-N_3AMP substituted RNAs can potentially be used to monitor siRNA localization and for studying the mechanism of siRNA mediate gene silencing.

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INTRODUCTION

Carcinoembryonic antigen (CEA) is a widely used tumor marker, which is over-expressed in a variety of cancers, including cancer of the breast. Recent studies suggest that deregulated overexpression of CEA promotes tumorogenesis by inhibiting cell differentiation of many cell types (Eidelman et al., 1993; Screaton et al., 1997), and by preventing anoikis, a type of apoptotic program which destroys cells that lose contact with the extra cellular matrix (Ordonez et al., 2000). Significantly, down regulation of CEA in human HT29 colon cancer cells has been shown to inhibit tumor cell aggregation and the reduction in colony formation (Wirth et al., 2002). Although these findings indicate that CEA might have a role in tumor progression, if and how CEA promotes breast cancer is not known. We hypothesized that if deregulated over-expression of CEA is linked to breast cancer progression, downregulation of CEA expression via destruction of its mRNA may promote apoptosis or alter the expression of genes that are involved in apoptosis and cell cycle/proliferation. To investigate the relationship between overexpression of CEA and breast cancer progression, we employed RNA interference (RNAi) to downregulate CEA mRNA. RNAi (Fire et al., 1998; Fire, 1999; Hammond et al., 2001; Sharp, 2001; Zamore, 2001) is a recently discovered phenomenon, which induces sequence-specific gene silencing in response to exogenous double-stranded RNA (dsRNA).

BODY

Although the start date of this project was August 1, 2003, the postdoctoral fellow who performed the proposed experiments joined our lab in the second week of September, which left us behind the schedule by more than a month. We have successfully accomplished the proposed task 1. However, as detailed below, the completion of this task took more than the expected time, which is why the experiments proposed in task 2 are currently underway.

Task 1: To establish conditions for siRNA-mediated cleavage of CEA mRNA in MCF7 cells

Because there are no sets of rules that can predict a site within a gene that may be accessible for siRNA mediated gene silencing, successful silencing of a gene requires the designing and testing of different siRNAs.

(i) Designing and cloning of small hairpin RNAs (shRNAs) for downregulation of CEA mRNA

It is now well established that siRNA sequences cloned as short-hairpin RNAs (shRNAs) are processed into siRNAs by Dicer, an RNase-III like activity present in mammalian cells (Bernstein et al., 2001). In an attempt to silence CEA gene, we constructed a series of siRNA expression vectors in which 21-nt sense and antisense sequence (separated by 8-nt loop) against CEA gene were placed under the control of U6 promoter. As outlined in Fig. 1 (for Figures please see appendix 1), a PCR amplified U6 transcription cassette containing anti-CEA shRNA was cloned into pCR2.1 vector (Invitrogen) essentially according to the published protocol (Bertrand et al., 1997; Lee et al., 2002). Following siRNA expression vectors which target different sites within CEA mRNA were prepared, and the authenticity of these constructs was confirmed by sequencing.

1. pShRNA53: homologous to CEA mRNA position 89-109*
2. pShRNA54: homologous to CEA mRNA 153-173*
3. pShRNA55: homologous to CEA mRNA position 191-211*
4. pShRNA56: homologous to CEA mRNA position 475-495*
5. pU6ShRev: this construct was originally designed to knockdown HIV-1 rev mRNA and will serve as a negative control.

*The CEA mRNA is numbered with respect to the translation initiation codon (ATG) with underlined adenosine as nucleotide 1.

(ii) To examine the ability of expressed siRNAs to silence CEA

To test the ability of siRNAs to knockdown CEA mRNA, MCF7 cells were transiently transfected with siRNA expression plasmids with Lipofectamine Plus reagent following manufacturer's instructions (Invitrogen). Briefly, MCF7 cells (obtained from ATCC, Rockville, MD) were grown and passed in DMEM medium supplemented with 10% fetal calf serum (FCS). Cells were seeded (24 h before transfection) at a density of $4x10^5$ cells/well in a 6-well plate at 50-70% confluency without antibiotic. After transfection and continued incubation at 37 ℃ for 48 h, cells were harvested and total RNA was isolated using Trizol reagent (Qiagen). Next, an RT-PCR assay was performed to monitor the levels of CEA mRNA. The primer set selected for RT-PCR has been shown to be highly CEA-specific, spanning large intron sequences, which avoids false positive results (Goeminne et al., 1999). The results shown in Fig. 2 indicate that apparently none of the expressed siRNAs was able to downregulate CEA mRNA. As discussed below, a number of reasons could account for this observation. First, the failure to uptake siRNA expression plasmids by MCF7 cells could account for the observed poor efficacy of anti-CEA siRNAs; low transfection efficiency of MCF7 cells has been well documented (Holle et al., 2004 and this study). Second, there is a possibility that the RNAi machinery failed to process shRNAs to 21-mer siRNAs. Third, the targeted sequence may be bound by cellular proteins and thus may not be accessible to the siRNA. Finally, it is important to remember that mRNA levels may not directly reflect changes in the level of expressed protein, especially if the hairpins are expressed in a transient setting (Paddison et al., 2004). In order to address these issues, we decided to undertake following experiments.

To monitor the cellular uptake, shRNAs were subcloned into a GFP reporter vector, pQBI (Qbiogene). To this end, U6 transcription cassettes were PCR amplified with forward and reverse primers containing *Bgl* II and *Xho* I sites, respectively (Fig. 3). After digestion with *Bgl* II and *Xho* I, the PCR amplified U6 cassettes were cloned into *Bgl* II/*Xho* I digested pQBI to yield pQBShRNA53, pQBShRNA54, pQBShRNA55, pQBShRNA56 and pQBscrambShRNA (a nonsilencing control) (Fig. 3). Because both GFP and the shRNAs are encoded by the same plasmid, although under the control of different promoters, GFP positive cells are expected to generate shRNAs. Transient transfections of HEK293 cells with these plasmids demonstrate the expression of GFP (Fig. 4). Similar results were obtained with MCF7 cells except that the transfection efficiency was between 25-30%. This suggests that uptake of shRNA expression vectors by MCF7 cells may not be the reason for poor efficacy of expressed siRNAs.

To determine whether the RNAi machinery processed shRNAs to 21-nt siRNAs, Northern analysis was performed. Briefly, HEK293 cells were transfected with shRNA expression plasmid and total RNA was isolated using Trizol reagent following manufacturer's instructions (Qiagen). Five micrograms of total RNA were fractionated in 6% polyacrylamide denaturing gel, and transferred onto Hybond-N+ membrane (Amersham Pharmacia Biotech). A ^{32}P-labelled 21-mer-oligonucleotide probe complementary to the siRNA-antisense was used for the hybridization reactions, which was performed for 16 h at 37 °C. The data shown in Fig. 5 indicates that all of the shRNAs were processed into 21-mer siRNAs, and thus rule out the possibility that failure to process shRNAs to siRNAs be the likely reason for the lack of CEA knockdown.

As a more direct approach to examine the ability of expressed siRNAs to knockdown CEA, we decided to analyze the levels of CEA protein using Western blot analysis. As shown in Fig. 6A and B, at 72 h post-transfection none of the siRNA expression transformants appears to lower the level of CEA protein as compared to the non-silencing control. At 96 h, however, siRNA#55 exhibits lower levels of CEA; compared to the control a 50% downregulation in the expression of CEA is observed (Fig. 6 C and D). Whereas siRNAs#53 and 56 has some effect (~20% reduction in CEA level), siRNA#54 could not elicit any effect. As a final test to assess whether expressed

siRNAs can knockdown CEA mRNA, we decided to assay siRNA mediate cleavage of CEA in HEK293 cells, a cell line known to have excellent transfection efficiency. Because HEK293 cells do not express endogenous CEA, cells were transfected transiently with pQBCEA-GFP, which overexpresses CEA as a GFP fusion. Cotransfection of pQBCEA-GFP and siRNA expression vector or nonsilencing control in HEK293 cells indicates that expressed siRNA could downregulate CEA mRNA (Fig. 7). Experiments are underway to investigate why expressed siRNAs were not efficient in downregulating endogenously expressed CEA, but could suppress exogenously expressed CEA.

(iii) Transfection of MCF7 cells with chemically synthesized siRNAs, verification of uptake and confirmation of downregulation of CEA

A recently published report involving the use of chemically synthesized siRNA to downregulate an endogenous gene in MCF7 cells suggests that chemically synthesized siRNA might serve as a better substrate for RNAi response (Chen et al., 2004). Thus, two siRNAs homologous to CEA mRNA from 402-422 (siRNA#497) and from 1948-1968 (siRNA#2043) were chemically synthesized (Qiagen). To monitor the cellular uptake, nonsilencing control siRNA was labeled with fluoresceine at the 5' end of the antisense strand. One day prior to transfection, MCF7 cells ($4x10^5$ cells/well) were seeded in 6-well plates containing DMEM medium without antibiotics. At 50-60% confluency, cells were transfected with chemically synthesized siRNA using Oligofectamine reagent following manufacture's protocol (Invitrogen). The final concentrations of siRNAs were 100 and 200 nM. To monitor the transfection efficiency cells were subjected to fluorescence microscopy (Olympus LH50A microscope). Transfection efficiency between 35-40% was routinely observed.

To assess the ability of chemically synthesized siRNAs to downregulate CEA, siRNA transfected MCF7 cells were incubated at 37 ℃ for 24, 48 and 72 h at which time cells were lysed following published protocol (Frick & Goodman, 1992), and the resulting lysate was cleared by centrifugation. Proteins were resolved on a 4-14% SDS-polyacrylamide gradient gel (Bio-Rad), transferred to polyvinylidene fluoride (PVDF) membrane and probed with anti-CEA antibody. As shown in Fig. 8, unlike nonsilencing control both of the siRNAs exhibit substantial downregulation of CEA protein with siRNA #497 more effective than siRNA #2043 (Fig. 8B and D). At this stage we do not know why at 72 h the higher concentration of siRNA#2043 failed to elicit any effect (Fig. 8D).

(iv) 8-N_3AMP substituted RNA: a potential tool for the labeling of siRNAs

The observation that chemically synthesized siRNAs can be employed to inhibit gene expression in mammalian cells has made siRNA an important tool to study gene function (Elbashir et al., 2001). While examining the uptake of fluorescent-labeled siRNAs by MCF7 cells, we realized that although such siRNAs could be obtained commercially, the cost of such a siRNA is more than $600. As an alternative, we have developed T7 RNA polymerase dependent in vitro transcription protocol that permits the incorporation of 8-N_3AMP into RNA. 8-N_3ATP is photoactivatable ATP analog, which has been widely used to map the nucleotide-binding site of a variety of proteins. The azide group of 8-N_3-adenosine substituted siRNA could be converted to a primary amine, which in turn may be conjugated to a variety of reporters, including fluorescent probes. The proposed approach could be used to prepare fluorescent-labeled siRNA, which can be used to monitor siRNA localization and for studying the mechanism of siRNA mediate gene silencing. This work has been accepted for publication in the journal *RNA*.

KEY RESEARCH ACCOMPLISHEMENTS:

- Designing, cloning and characterization of anti-CEA siRNAs

- Subcloning of anti-CEA siRNAs in GFP expression vectors
- Confirming the processing of expressed shRNAs to 21-mer siRNAs
- Construction and characterization of plasmid expressing CEA-GFP fusion protein
- Designing of two chemically synthesized anti-CEA siRNAs
- Confirming the silencing of CEA with chemically synthesized siRNAs
- Development of a new approach for template dependent incorporation of 8-N_3AMP into RNA

REPORTABLE OUTCOMES:

A manuscript entitled "Template-Dependent Incorporation of 8-N_3AMP into RNA with Bacteriophage T7 RNA Polymerase" has been accepted for publication in the journal *RNA*.

CONCLUSIONS:

- Identification of a suitable site within a gene of interest that could be targeted by siRNA requires designing and testing of different siRNAs.
- An siRNA that can knockdown the expression of an exogenous gene may not necessarily silence the same gene when expressed endogenously.
- A chemically synthesized siRNA homologous to CEA (nucleotide 402-422) has been most effective in suppressing the expression of CEA.
- Under modified transcription conditions T7 RNA polymerase can accept 8-N_3ATP as a substrate, and 8-N_3AMP substituted RNAs can potentially be used to monitor siRNA localization and for studying the mechanism of siRNA mediate gene silencing.

FUTURE GOALS:

1. To analyze gene expression profiling of MCF7 cells transfected with siRNA#497 and an irrelevant control. The results of microarray experiments will allow us to identify genes that, by virtue of their relationship to CEA, may be involved in tumorogenesis. For example, if the microarray data indicates that reduction in CEA expression is associated with an altered level of bcl-2 expression, we will quantitate bcl-2 using a bcl-2 ELISA kit (Oncogene Research). In parallel an RT-PCR using the human bcl-2 primer pair will be conducted.

2. To investigate the effect of CEA downregulation on apoptosis or cell cycle/proliferation. MCF7 cells transfected with siRNA#497 will be analyzed for apoptosis and cell cycle/proliferation. Because the initial results of apoptotic signaling are intracellular when the plasma membrane remains intact, we will use, in addition to TUNEL, biochemical assays that can detect solubilized Nuclear Matrix Proteins (NMPs), caspase activation, and increase in Fas levels. The tumor cells can expand in number either by bypassing apoptosis or by increasing the rate of cell proliferation. In some cases, inhibition of apoptosis also suppresses proliferation, normally associated with the up-regulation of bcl-2. Cells with up-regulated bcl-2 avoid entering the cell cycle, unless bax, the bcl-2 antagonist is activated. Thus, to better understand the effect of CEA cleavage on cell cycle progression/proliferation, siRNA treated MCF7 cells will be analyzed for cell cycle/proliferation markers. For example, we will specifically ask if the levels of p53, proliferating cell nuclear antigen (PCNA), $p21^{WAF1}$ family, BAX, Cyclin A, D or D3 are different among the cell population in which the CEA is down regulated. Kits (Oncogene Research, Germany) that can quantify the levels of these markers will be used.

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Appendix 1

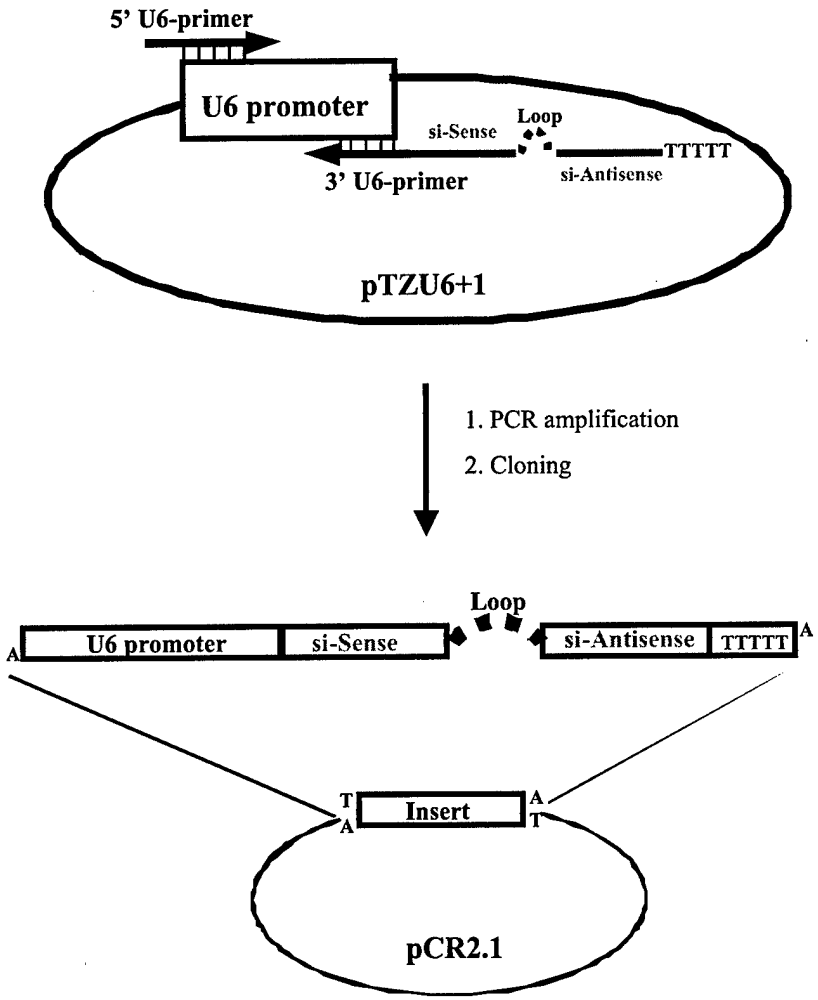


Figure 1: Schematic representation of the PCR strategy used to generate siRNA expression plasmids. The 5' PCR primer is complementary to the 5' end of the U6 promoter and the 3' PCR primer is complementary to the sequences at the 3' end of the U6 promoter followed by the sense, loop, antisense and a stretch of five to six deoxyadenosine as termination signal for the U6 Pol III promoter driven transcription. The plasmid pTZU6+1, which contains U6 promoter, is used as a template for PCR amplification. The PCR amplified U6 transcription cassette was cloned into pCR2.1 vector using TA cloning kit (Invitrogen).

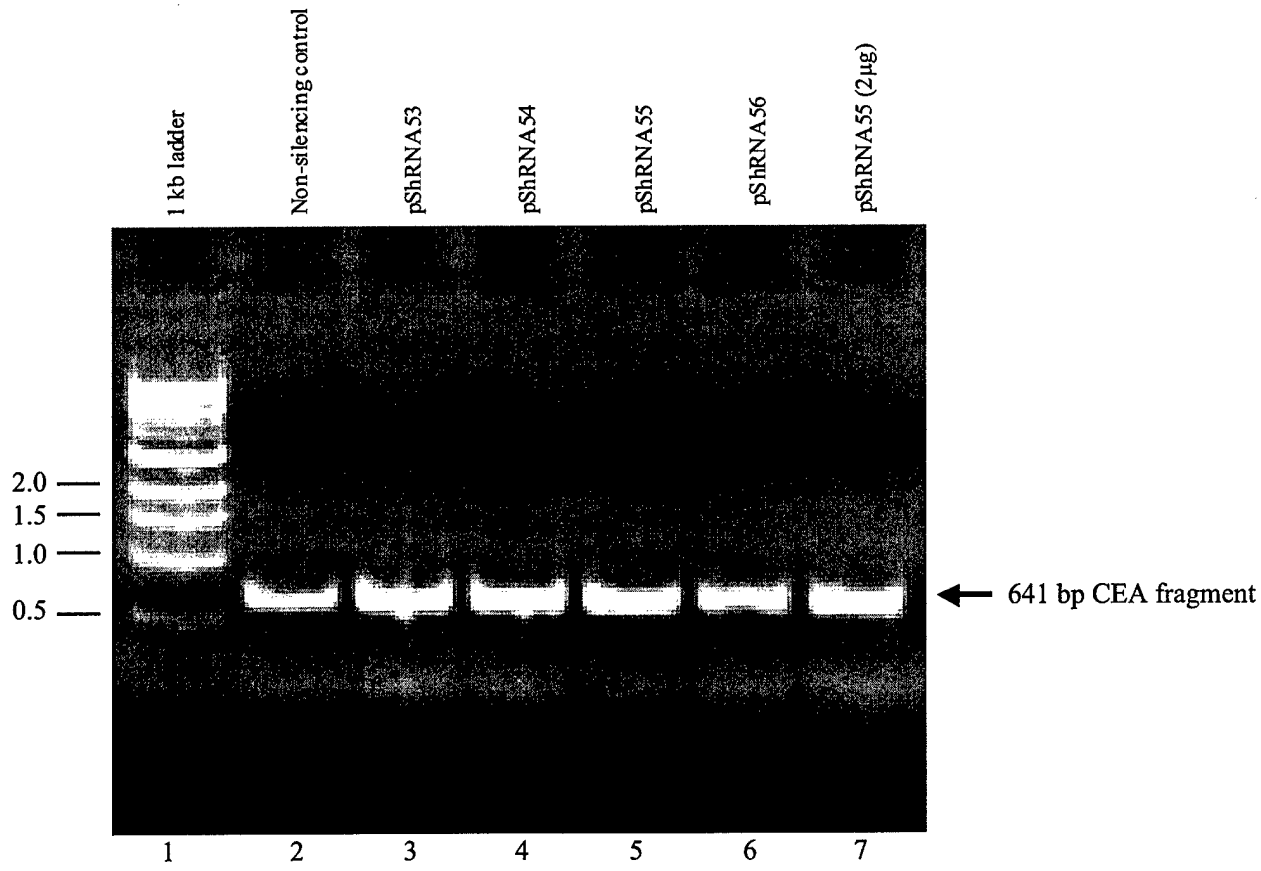


Figure 2: RT-PCR analysis of CEA mRNA isolated from MCF7 cells (48 h post-transfection), which were transiently transfected with pU6ShRev (nonsilencing control) or with pShRNA53-56 (plasmids expressing anti-CEA siRNAs). After RT-PCR with forward (5'-CCA TGG AGT CTC CCT CG-3') and reverse primer (5'-GTA GCT TGC TGT GTC ATT TC-3'), the products (lanes 3-7 siRNA treated, and lane 2 negative control) were analyzed on a 1% agarose gel with ethidium bromide staining. The expected 640 bp CEA fragment is indicated.

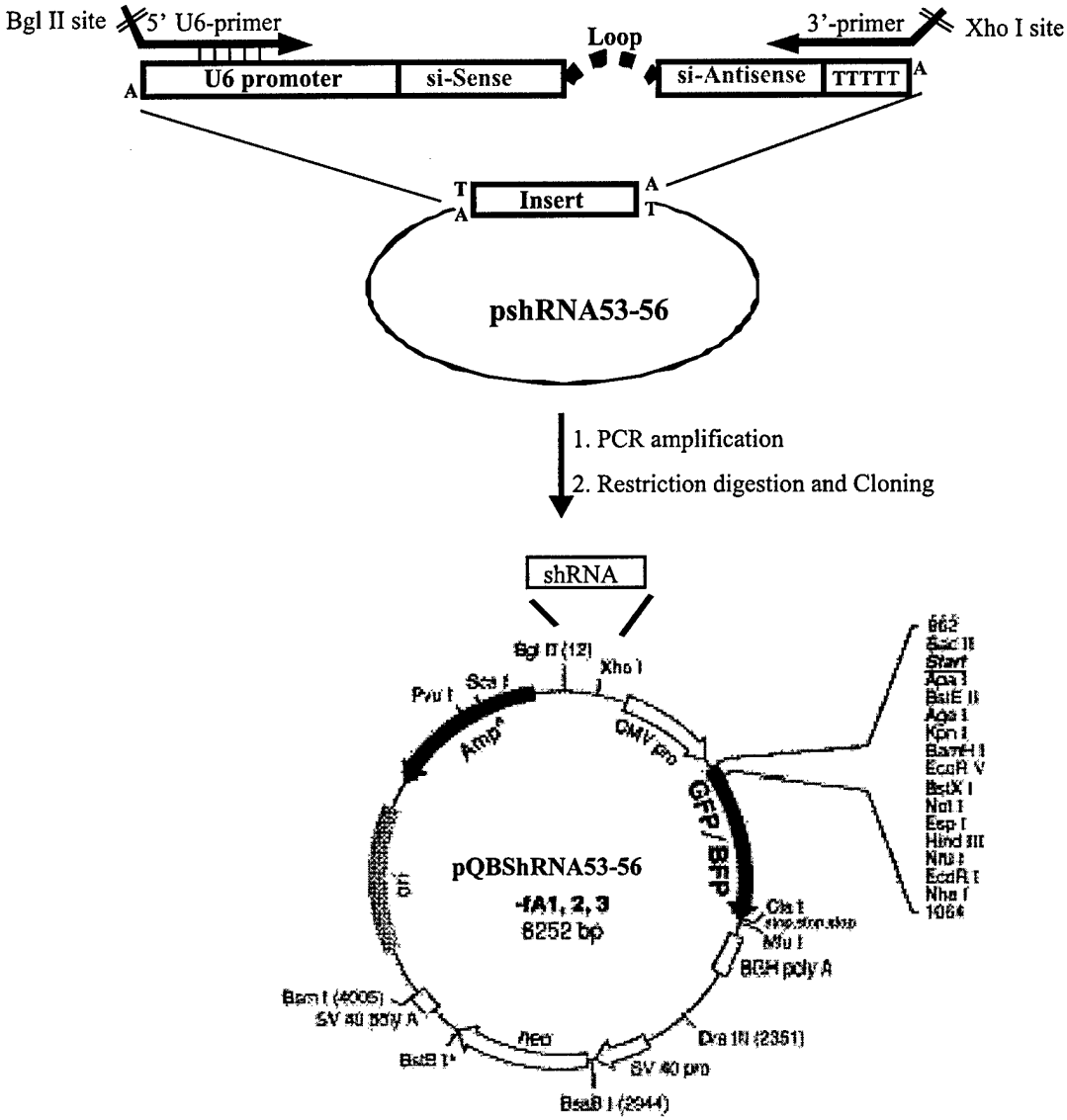
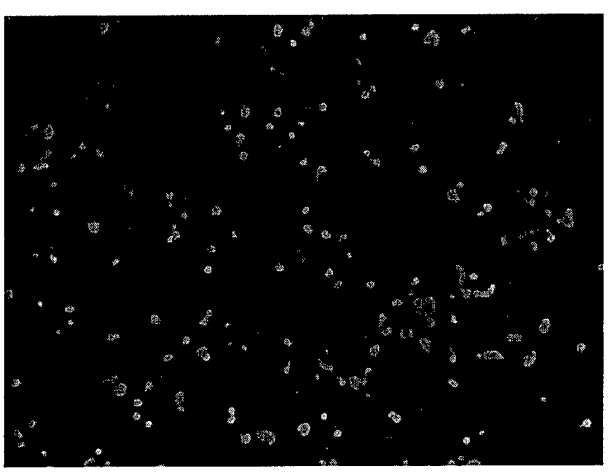
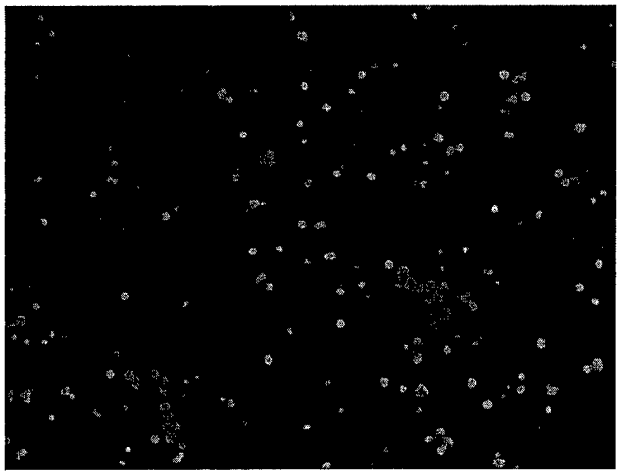


Figure 3: Schematic representation of the strategy employed for the construction of plasmids pQBShRNA53-56. The 5' PCR primer is complementary to the 5' end of the U6 promoter and the 3' PCR primer is complementary to the sequences at the 3' end of the shRNA. PCR amplified DNA was digested with *Bgl* II and *Xho* I and cloned into pQBI, which was digested with *Bgl* II/*Xho* I.



pQBscrambShRNA



pQBShRNA55

Figure 4: Fluorescence microscopy to monitor the uptake of siRNA expressing plasmid pQBShRNA55 or nonsilencing control by HEK293 cells. HEK293 cells were transiently transfected with 2.0 μg of plasmid. Northern analysis (as described in Fig. 5) confirmed the expression of siRNA.

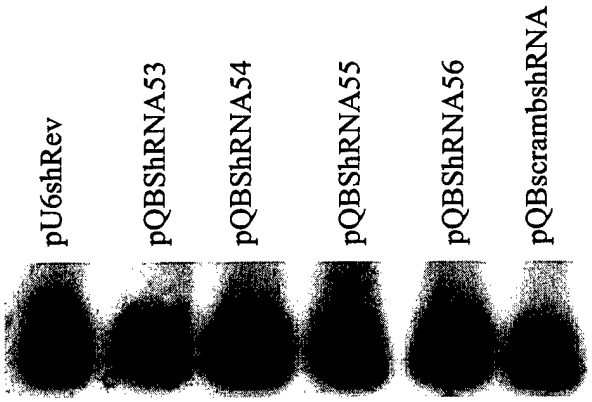


Figure 5: The expressed shRNAs are processed to 21-mer siRNAs. Northern gel analyses of siRNAs expressed from indicated plasmids transfected in HEK293 cells. The oligonucleotide probes are complementary to the antisense strand of siRNAs.

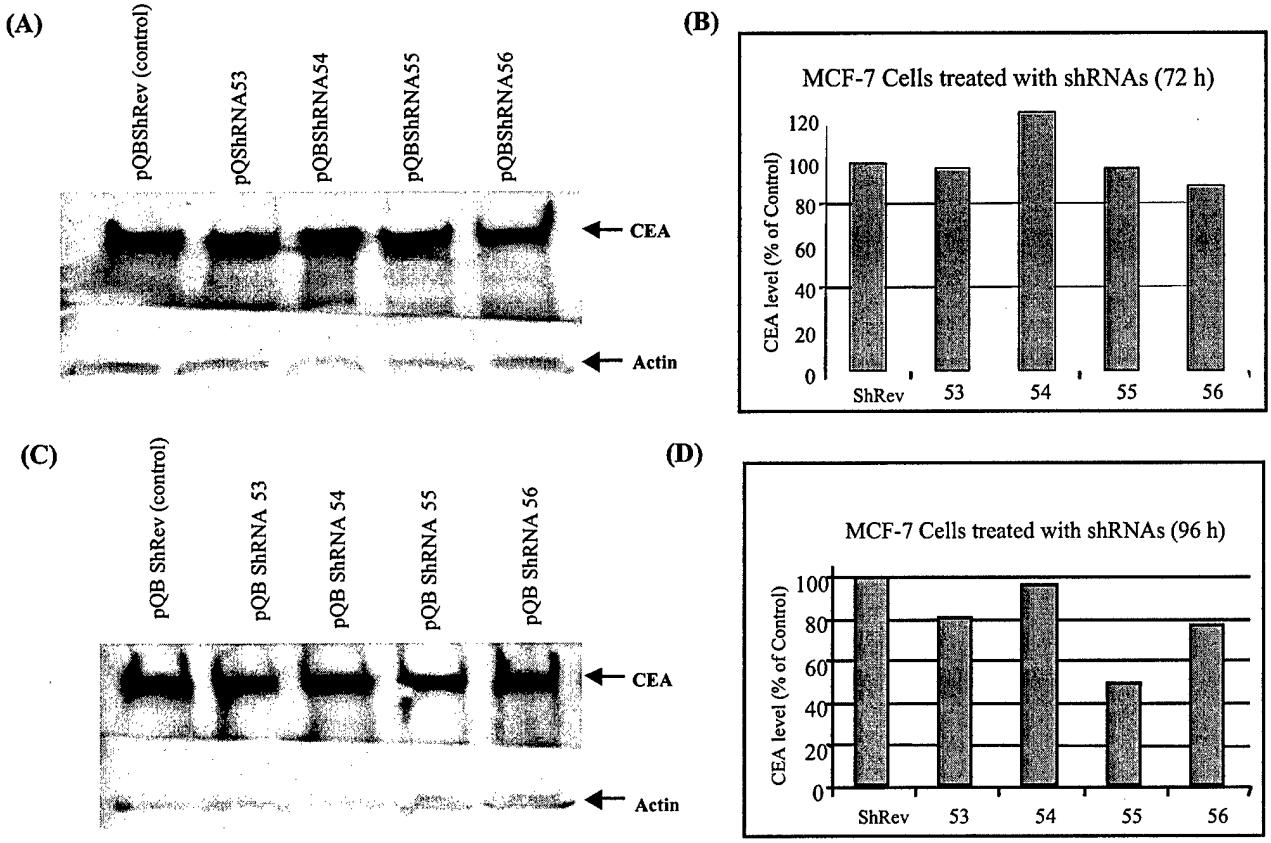


Figure 6: Effects of the expressed siRNAs on the level of endogenous CEA. MCF7 cells were transiently transfected with the indicated plasmid and after 72 or 96 h cell lysate was prepared. Proteins were separated on a gradient SDS-polyacrylamide gel, transferred and probed with anti-CEA antibody.

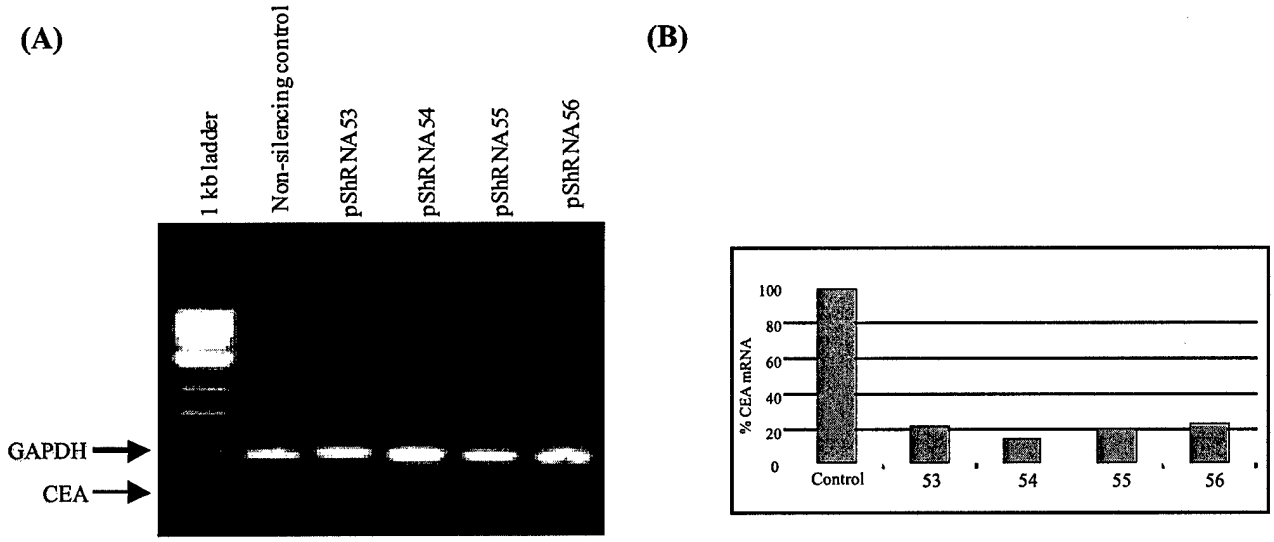


Figure 7: Expressed anti-CEA siRNAs can downregulate exogenously expressed CEA mRNA. HEK293 cells were cotransfected with pQBCEA-GFP and siRNA expression vector (pShRNA53-56) or nonsilencing control. After 48 h, total cellular RNA was isolated and subjected to RT-PCR with CEA or GAPDH specific primers.

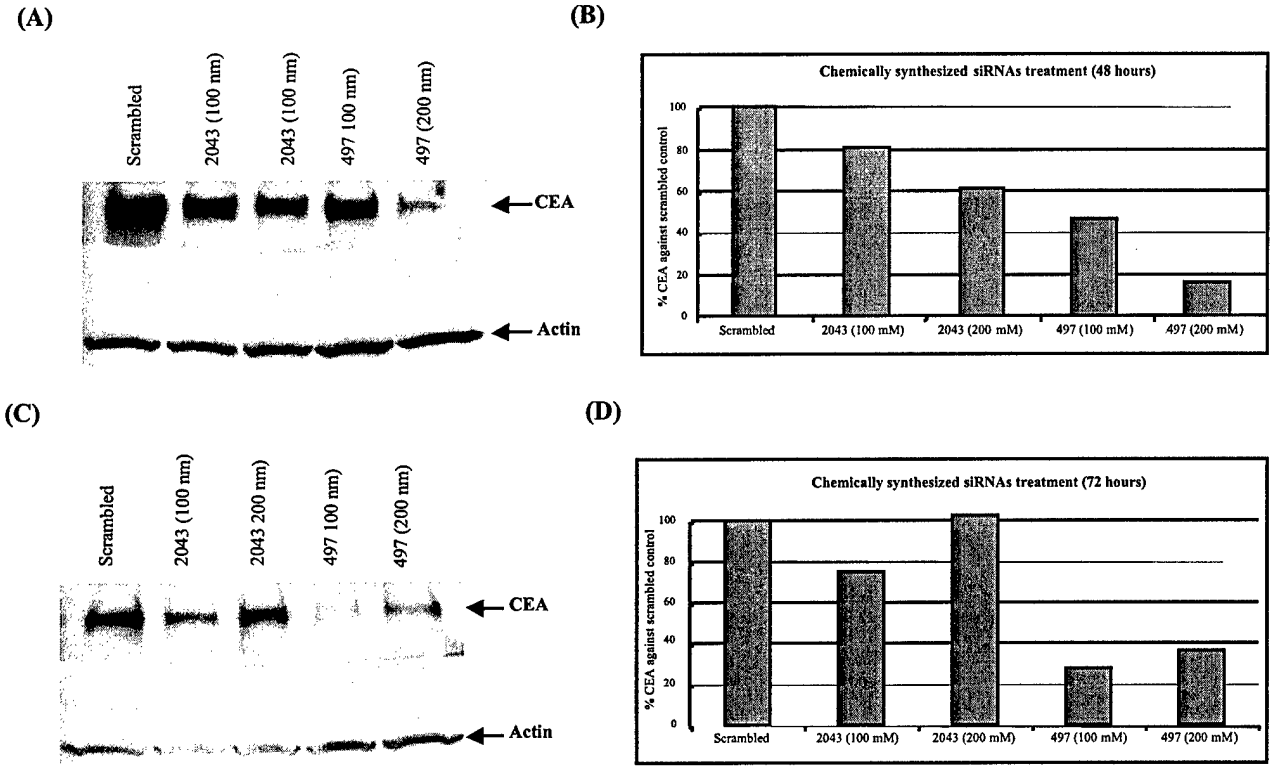


Figure 8: Effects of chemically synthesized siRNAs on the level of endogenous CEA. MCF7 cells were transfected with the indicated siRNA and after 48 or 72 h cell lysate was prepared. Proteins were separated on a gradient SDS-polyacrylamide gel, transferred and probed with anti-CEA antibody.